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COMPARATIVE CHROMATOGRAPHY OF LECTINS AND BIOACTIVITY RECOVERY OF THE IMMUNOLOGIC HORMONE LEUKOREGULIN ON DERIVATIZED SILICA AND ON CROSS-LINKED AGAROSE MOLECULAR SIZING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MATRICES

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SUMMARY

This investigation compares the performance of the new zirconia stabilized silica and cross-linked agarose size-exclusion matrices to Spherogel-TSK 3000 SWG silica in high-performance liquid chromatographic separation of proteins possessing a range of molecular weights present in many lymphokine preparations and in recovery of bioactivity as measured by leukoregulin proliferation inhibitory activity. Retention time *versus* log molecular weight of protein standards was linear from 12 500 to 290 000 on the agarose and from 32 000 to 290 000 on the other columns. Recovery of leukoregulin proliferation inhibitory activity directed against RPMI 2650 epidermoid carcinoma cells was 90% from the silica, 88% from the agarose and 35% from the zirconia stabilized silica columns.

INTRODUCTION

This investigation compares the performance of the newer zirconia stabilized silica and cross-linked agarose size-exclusion matrices to Spherogel-TSK 3000 SWG silica in separating proteins at physiological pH possessing a range of molecular weights present in many lymphokine preparations. It also analyzes the performance of the matrices with respect to recovery of macromolecular bioactivity as measured by the proliferation inhibitory activity of the lymphokine and immunologic hormone, leukoregulin[1].

True size-exclusion chromatography of macromolecules is best if not only achieved under column chromatographic conditions favoring near or complete denaturation of the biopolymers[2–4]. The separation of biologically active macromolecules, however, under conditions conductive to preserving biological activity, *e.g.* aqueous buffered solvents with reasonable ionic strength and pH, permits a variety of factors in addition to molecular size to influence the separation characteristics of a macromolecule. Electrostatic and adsorptive solute-stationary phase interactions

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and conformational changes in the macromolecule, for example, in addition to molecular size depending upon the size inclusion matrix influence the molecule's retention time in passing through the molecular sizing matrix within the column[4,5]. Solute–matrix interactions of this nature may be detrimental to achieving an accurate estimate of biopolymer molecular size based upon retention time. At the same time they permit separation of macromolecules of similar molecular size due to differences in the primary, secondary, tertiary and quaternary structures of the macromolecules which may be very useful and at times even critical to preparative size-exclusion high-performance liquid chromatography (HPLC) of bioactive polymers.

Chronic exposure of the silica matrix within the column to a pH above 7 results in solubilization and progressive destruction of the silica matrix accompanied by diminution in the size-exclusion resolving power of the column[6,7]. Recently prepacked size-exclusion HPLC columns containing stabilized silica or cross-linked agarose matrices have become commercially available. These materials should be unaffected by repeated exposure to buffers with pH's greater than 7 when such conditions are required for purification of biologically active macromolecules. Size-exclusion HPLC columns containing matrices of these materials if they possess equal or better resolving power than columns containing "non-stabilized" silica would be considerably more economical for long-term use in the purification of bioactive biopolymers.

In the present investigation columns were calibrated with commercially prepared HPLC molecular weight (MW) protein standards. The relative molecular sizing separation characteristics of the columns were assessed with lectins, sugar-binding proteins or glycoproteins of non-immune origin capable of agglutinating erythrocytes and certain other cells by binding to specific carbohydrate receptor sites on cell surfaces[8,9]. Lectins were selected as they are commercially available as preparations containing a diversity of molecular sized monomeric and oligomeric forms[10–19] and their chromatographic profile on the diverse HPLC matrices would provide a comparison of the column performances in separating heterogeneous glycoprotein samples at a physiological pH. The molecular heterogeneity present in the lectin preparations was assessed by non-dissociating polyacrylamide gel electrophoresis (PAGE) and by dissociating-sodium dodecyl sulfate (SDS)-PAGE as a reference for comparing the elution profiles of the lectins on the three HPLC column matrices. Recovery of leukoregulin bioactivity from the silica, zirconized stasbilized silica and agarose matrices was compared using conditions previously demonstrated with the silica matrix to yield 90% recovery of the macromolecular [HPLC average molecular weight (MW_{avg}) approximately 50 000] leukoregulin bioactivity[1].

MATERIALS AND METHODS

Chromatography columns

Three commercially pre-packed columns were studied in this investigation. An Altex Spherogel-TSK 3000 SWG 600 mm × 21.5 mm I.D. column with a 75 mm Spherogel-TSK SWG precolumn was obtained from Beckman Instruments (Berkeley, CA, U.S.A.). The Spherogel-TSK 3000 SWG column is packed with rigid hydrophilic (alcohol derivatized) spherical 13 ± 2 μm silica particles suitable for separation according to the manufacturer's product literature of biopolymers with MW

of 1000 to 300000. A Superose 12 HR 10/30 300 mm × 16 mm I.D. column was obtained from Pharmacia, (Piscataway, NJ, U.S.A.). This column is packed with a cross-linked agarose based matrix of 10 ± 2 μm particles suitable for separation according to the manufacturer's product literature of biopolymers in the MW range of 1000 to 300000. A Zorbax GF-250 250 mm × 9.4 mm I.D. column preceded by a Zorbax precolumn and guard column packed with 20 μm bonded Zorbax silica was obtained from the DuPont (Wilmington, DE, U.S.A.). The Zorbax column contains a Zirconia clad silica support packing of 4–5 μm spherical particles modified with a hydrophilic organosilane compound to produce a homogeneously bonded monomeric hydrophilic (di-alcohol) stationary phase for separation according to the manufacturer's product literature of biopolymers with MW in the range from 10000 to 250000.

Chromatography system

The columns were interconnected at the inlet end to a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump and U6K sample injector with a 2-ml injection loop with the effluent delivered to a Waters Model 440 spectrophotometer set to measure absorbance at 280 nm. A Waters Model 730 data module and M721 system controller were used to graphically record effluent absorbance and retention time. The Spherogel-TSK 3000 SWG column was eluted as previously described[1] at 4 ml/min and 375 p.s.i. and 100-μl samples were applied to the column. The Zorbax GF-250 column was eluted at 1 ml/min and 600 p.s.i. and 25-μl samples were applied to the column. These elution and sample application volumes were selected to be within the range of up to 2 ml/min and 200 μl suggested by the column manufacturer to achieve optimal separation. The Superose 12 column was eluted at 0.5 ml/min and 425 p.s.i. and 50-μl samples were applied to the column. These elution and sample application volumes were selected to be within the range of up to 1.0 ml/min and 100 μl suggested by the column manufacturer to achieve optimal separation. Elution of protein standards, lectins and leukoregulin was carried out at room temperature.

Buffers

The columns were eluted with 0.02 *M* sodium phosphate, pH 7.4, with and without 0.1% MW 3000 polyethylene glycol (Sigma, St. Louis, MO, U.S.A.)[1], 0.1 *M* sodium phosphate, pH 7.4, with or without 0.1% polyethylene glycol or with 0.01 *M* sodium phosphate–0.14 *M* sodium chloride, pH 7.4, with or without 0.1% polyethylene glycol.

Proteins

HPLC protein standards in the form of a MW (HPLC) Marker Protein Kit were obtained from the United States Biochemical Corp. (Cleveland, OH, U.S.A.). Each kit contained 200-μg protein consisting of MW 12000 cytochrome *c*, MW 32000 adenylate kinase, MW 67000 enolase, MW 140000 lactic dehydrogenase and MW 290000 glutamic dehydrogenase. The contents of one vial were dissolved in the buffer used to elute the column and applied to the column within 24 h. Concanavalin A, fava bean, gorse, hairy vetch, lotus, Phytohemagglutinin-L, peanut, pokeweed, soybean, and wheatgerm lectins were purchased from Sigma and were dissolved in the buffer used to elute the column. Leukoregulin was prepared and assayed as pre-

viously described[20] and was equilibrated prior to injection with the buffer used to elute the column. As previously reported 90% or more of leukoregulin activity can be recovered when sample volumes of as much as 2 ml are applied to the Spherogel-TSK 3000 SWG column and eluted at 4 ml/min with 0.02 *M* sodium phosphate–0.1% MW 3000 polyethylene glycol, pH 7.4 buffer[1]. All samples were filtered through a 0.22-μm Millex filter (Millipore, Bedford, MA, U.S.A.) prior to administration to the U6K injector.

Polyacrylamide gel electrophoresis

Analytical non-dissociating PAGE was performed in a 1.5 mm thick × 160 mm long 10.0% polyacrylamide gel with a 2.5% stacking gel in a non-dissociating discontinuous 0.0625 *M* Tris-HCl, pH 6.8, buffer system[21]. Lectins and the MW marker proteins for non-denatured PAGE (Sigma) including α-lactalbumin (MW 14200), bovine carbonic anhydrase (MW 29000), egg albumin (MW 45000), bovine serum albumin (MW 66000 monomer and 132000 dimer), and Jack Bean urease (MW 240000 monomer and 480000 dimer) were dissolved in 10% glycerol–0.062 *M* Tris-HCl, pH 6.8. Electrophoresis was carried out at 70 V for approximastely 16 h at 4°C. Gels were dried and stained with an aqueous solution containing 0.1% Coomassie blue, 25% methanol and 15% acetic acid. Dissociating-SDS-PAGE was performed in 12.5% polyacrylamide gel with a 2.5% stacking gel with 2% SDS and 1% dithiothreitol added to the sample equilibrium and running 0.0625 *M* Tris–HCl, pH 6.8, buffer[22]. Samples in the equilibrium buffers were heated at 100°C for 5 min before application to the dissociating gel. Each gel lane was inoculated with up to 60 μg protein. Lectins, Dalton Mark VII-L molecular weight marker proteins for SDS gel electrophoresis (Sigma) including α-lactalbumin (MW 142000), soybean trypsin inhibitor (MW 20100), trypsinogen (MW 24000), bovine carbonic anhydrasse (MW 29000), glyceraldehyde-3-phosphate dehydrogenase (MW 36000), egg albumin (MW 45000), and bovine serum albumin (MW 66000), immunoglobulin G (MW 150000) (Sigma), and myosin (MW 205000) (Sigma) were electrophoresed at 70 V for 16 h at 4°C. Gels were dried and stained with Coomassie blue to visualize the protein bands in the same manner as in the non-dissociating gels. The relative position of each band in the lectin samples relative the MW protein standards was determined and used in calculating the PAGE MW_{avg} of the components in the 10 lectin preparations.

RESULTS

The silica, zirconia stabilized silica and the cross-linked agarose matrices each resolved the mixture of MW HPLC marker protein standards into five or more peaks. Typical 280 nm absorbance profiles and peak retention times are shown in Fig. 1 which illustrates that the widest separation of the individual proteins in the MW range from 10000 to 300000 was obtained on the TSK 3000 SWG silica column with slightly less resolution on the Superose 12 cross-linked agarose column. The separations were similar in the presence or absence of MW 3000 polyethylene glycol on the TSK 3000 SWG and Superose 12 columns. The resolution of the proteins in the MW marker protein mixture was less distinct and was affected in addition by the polyethylene glycol on the Zorbax GF-250 zirconia stabilized silica column (Fig. 2).

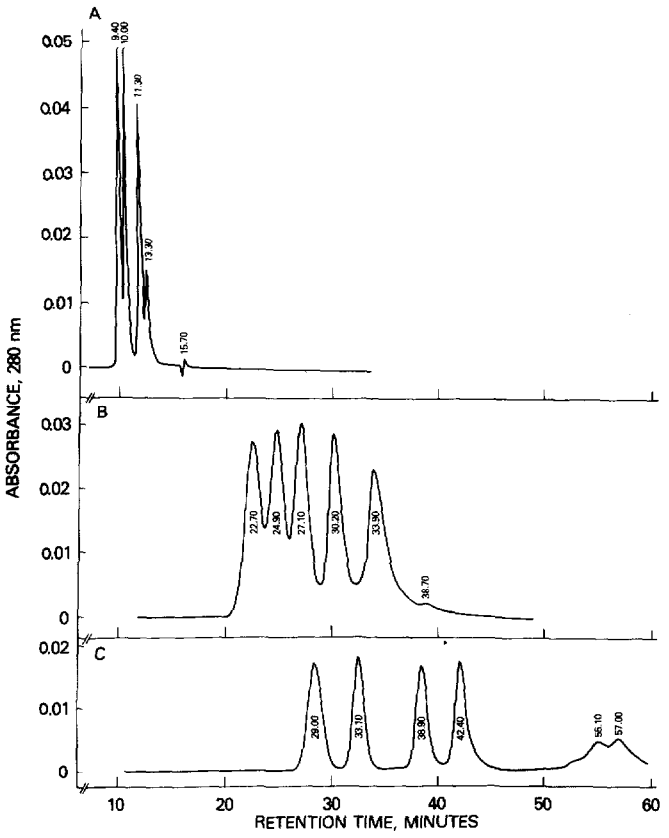

Fig. 1. Separation of a mixture of MW HPLC marker protein standards on (A) Zorbax GF-250 zirconia clad silica, (B) Superose 12 cross-linked agarose and (C) Spherogel-TSK 3000 SWG silica molecular sizing HPLC columns. A volume of 25 μl (20 μg) of the protein standards in 0.1 *M* sodium phosphate, pH 7.4–0.1% MW 3000 polyethylene glycol was applied to the GF-250 column and eluted at 1 ml/min. A volume of 50 μl (50 μg) of the protein standards in 0.01 *M* sodium phosphate–0.14 *M* sodium chloride, pH 7.4–0.1% MW 3000 polyethylene glycol was applied to the Superose 12 column and eluted at 0.5 ml/min. A volume of 100 μl (100 μg) of the protein standards in 0.02 *M* sodium phosphate, pH 7.4–0.1% MW 3000 polyethylene glycol was applied to the Spherogel-TSK 3000 SWG column and eluted at 4 ml/min. Absorbance was measured by a Waters Model 440 spectrophotometer at 0.05 a.u.f.s. and the retention times (recorded above the peaks) obtained by a Waters Model 730 data module. The five peaks in Fig. 1A correspond to: MW 290 000 glutamic dehydrogenase, 9.40 min retention time; MW 140 000 lactic dehydrogenase, 10.00 min; MW 67 000 enolase, 11.30 min; MW 32 000 adenylate kinase, 13.30 min; and MW 12 400 cytochrome *c*, 15.70 min. The additional peaks with retention times of 38.70 min in (B) and 57.00 min in (C) are contaminants present in the marker protein mixture; the earlier peaks are the same as in (A).

Nevertheless an essentially linear relationship between log MW *versus* retention time was obtained from 12 500 to 290 000 for the cross-linked agarose size-exclusion column and from 32 000 to 290 000 on the silica and zirconia clad silica columns (Fig. 2).

The characteristics of the three different MW calibrated size-exclusion matrices in resolving diverse molecular size proteins were analyzed by comparing the sepa-

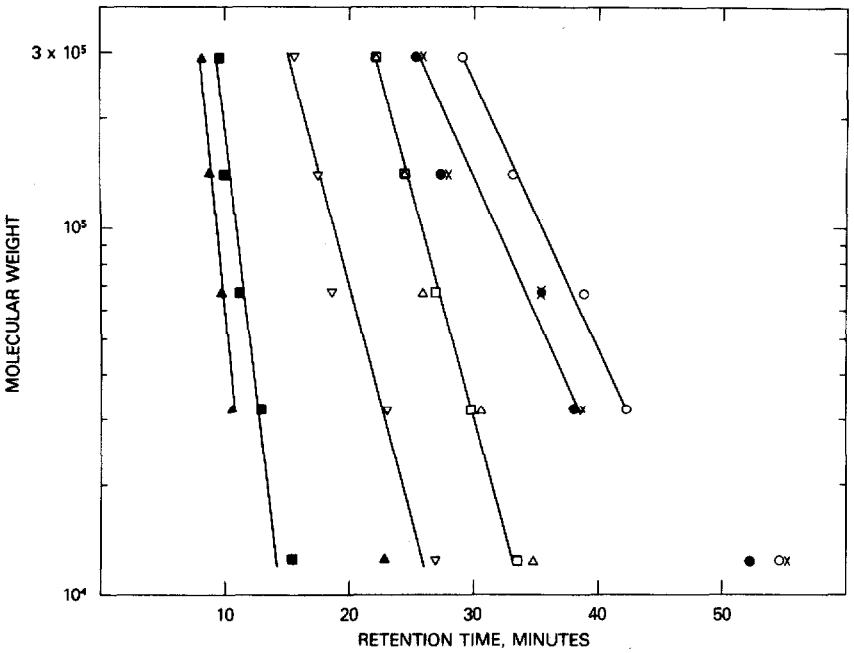

Fig. 2. Molecular weight calibration curves for the Zorbax GF-250 (▲, ■, ▽), Superose 12 (△, □) and Spherogel-TSK 3000 SWG (○, ●, ×) HPLC columns using the MW marker protein standard mixture (Fig. 1) in the presence and absence of 0.1% MW 3000 polyethylene glycol in the samples and elution buffers. The columns were eluted with 0.02 *M* sodium phosphate, pH 7.4, with (×) and without (●) polyethylene glycol; with 0.1 *M* sodium phosphate with (▽) and without (▲) polyethylene glycol and with 0.01 *M* sodium phosphate–0.14 *M* sodium chloride, pH 7.4, with (○, △, ■) and without (□) polyethylene glycol. The void volumes for the columns as determined by the elution of blue dextran were 105.6 ml for the TSK 3000 SWG column, 7.5 ml for the Superose 12 column and 8.6 ml for the Zorbax GF-250 column in 0.01 *M* sodium phosphate–0.14 *M* sodium chloride, pH 7.4–0.1% MW 3000 polyethylene glycol.

ration profiles of ten plant lectins. Chromatography of the ten lectins on the silica, zirconia stabilized silica and cross-linked agarose matrix columns yielded distinctly different separation patterns on the three columns (Table I). The molecular size distribution patterns for a given lectin on the three HPLC columns were frequently within the MW range indicated by PAGE for the lectin even though the number and size of the column components diverged widely from those indicated by non-dissociating and dissociating-SDS-PAGE. This was true for lectin preparations where a single component was identified on each column, *e.g.* hairy vetch and peanut lectin, as well as for lectins where more than one component was identified on one or more of the three columns, *e.g.* phytohemagglutinin-L and pokeweed lectins. In some cases, however, both larger, *e.g.* fava bean, gorse, and pokeweed lectins, and smaller, *e.g.* lotus, phytomagglutinin-L, pokeweed, soybean, and wheat germ lectin, components were present in the HPLC profiles. Concanavalin A was the only lectin preparation whose HPLC components all were within the range of molecular weights exhibited by the lectin on PAGE yet the separation profiles for concanavalin A on the three matrices were as diverse as those of any of the other nine lectin preparations.

TABLE I

COMPARATIVE SEPARATION OF PLANT LECTINS ON SILICA AND AGAROSE MATRIX HPLC MOLECULAR SIZING COLUMNS

Lectin	*Published MW*	*Electrophoretic MW distribution**		*HPLC column molecular size distribution***		
		PAGE	*SDS-PAGE*	*TSK 3000*	*Superose 12*	*ZorbaxGF-250*
Concanavalin A	102 000 (10)	68 000 and 106 000	18 000 and 36 000	100 000	70 000 (10) and 110 000 (90)	30 000
Fava bean	50 000 (11)	100 000	20 000 and 23 000	105 000	95 000	180 000 (20) 410 000 (80)
Gorse	170 000 (12)	100 000	38 000 and 40 250	30 000	61 000 (23) and 135 000 (77)	95 000
Hairy vetch	139 000 (13)	68 000 and 160 000	21 000 34 000 44 000	110 000	125 000	22 000
Lotus	58 000, 117 000 and 120 000 (14)	160 000 and 222 000	24 000 and 38 000	32 000 and 61 000	⩽10 000 (23) and 27 000 (77)	155 000
Phytohemagglutinin-L	68 000 (15)	68 000 and 180 000	37 000	71 000	⩽10 000 (46) 24 000 (20) 99 000 (34)	32 000 (35) 98 000 (65)
Peanut	120 000 (16)	260 000	37 500 and 46 000	61 000	105 000	145 000
Pokeweed	32 000 (17)	163 000	36 000, 52 500, and 120 000	13 500 (38) and 240 000 (62)	26 000 (40) and 70 000 (60)	53 000 (48) and 150 000 (52)
Soybean	110 00 and 120 000 (18)	100 000 and 200 000	37 500 and 42 000	10 500 (14) 66 000 (16), 100 000 (52) and 125 000 (12)	115 000	200 000
Wheatgerm	36 000 and 123 500 (19)	360 000	20 000 and 31 500	71 000	⩽10 000 (50) and 100 000 (50)	36 000

* The molecular size distribution present in commercial lectin samples was analyzed by PAGE and SDS-PAGE and the molecular weights of the Coomassie blue stained bands calculated in reference to MW marker protein standards electrophoresed on the same gel. When more than one major band was present the molecular weight of each is listed.

** Each HPLC column was eluted with 0.01 *M* sodium phosphate–0.14 *M* sodium chloride, pH 7.4, containing 0.1% MW 3000 polyethylene. Values shown are for peaks containing 10% or more of the total A_{280} with MW assigned based upon calibration of the columns with MW marker protein standards (Fig. 2). When more than one peak was present the percentage of the total absorbance at 280 nm is listed in parentheses after the molecular weight of the peak.

The recovery of leukoregulin bioactivity from the three size-exclusion columns was also dissimilar. Approximately 90% of the leukoregulin proliferation inhibitory activity for RPMI 2650 carcinoma cells was recovered from the TSK 3000 SWG

TABLE II

RECOVERY OF LEUKOREGULIN ACTIVITY FROM SPHEROGEL-TSK 3000 SWG SILICA, ZORBAX GF-250 ZIRCONIA STABILIZED SILICA, AND SUPEROSE-12 CROSS-LINKED AGAROSE MATRIX MOLECULAR SIZING HPLC COLUMNS

Leukoregulin containing 100 RPMI cytostatic units per ml 0.01 *M* sodium phosphate–0.14 *M* sodium chloride, pH 7.4–0.1% MW 3000 polyethylene glycol was applied to each column. Effluent fractions in the molecular weight range from 10000 to 150000 based upon previous chromatography of MW marker protein standards (see Fig. 2) were collected, pooled, concentrated by diafiltration and the presence of proliferation inhibitory activity directed against RPMI 2650 nasal septum epidermoid carcinoma cells quantitated by microcytotoxicity assay[1].

Column	*Bed size (length × I.D. in mm)*	*Sample volume (µl)*	*Flow-rate (ml/min)*	*Recovery of biological activity (%)*
Spherogel-TSK 3000 SWG	600 × 21.5	100	4	90
Zorbax GF-250	250 × 9.4	25	1	35
Superose 12	300 × 16	50	0.5	88

silica column and from the Superose 12 cross-linked agarose column (Table II). In comparison only 35% of the leukoregulin activity was present in the effluent obtained from the Zorbax GF-250 zirconia stabilized silica column.

DISCUSSION

Speed of separation makes preparative HPLC attractive as a means to isolate biologically active macromolecules particularly when biopolymer activity is sensitive to deviations from physiological conditions. The results of the present investigation demonstrate, however, that even though different preparative HPLC matrices may yield similar separations of selected MW marker proteins, very different patterns of separation and recovery of bioactivity may be obtained during chromatography of other proteins.

Separation of macromolecules in relation to their molecular size requires the molecules to percolate through a matrix resulting in retardation of the molecules in proportion to their effective molecular size. The retardation or increased retention time of biologically active macromolecules on the basis of molecular size depends upon the extent of penetration of the molecules into the porous particle matrix and frequently is also influenced by a diversity of macromolecular solute-matrix stationary phase electrostatic, adsorptive, and at times absorptive interactions. The extent and complexity of the interactions can be expected to increase, furthermore, as the solute and solvent approach physiologic conditions in terms of ionic strength and pH.

As the speed of separation increases so does the intra-column pressure necessitating a more rigid size-exclusion or molecular sizing matrix. Beaded or spherical silica has proven to be a reliable matrix for HPLC column separations. The presence of the derivatized alcohol groups to increase the hydrophilic nature of the silica, however, can lead to significant solute–stationary phase electrostatic and adsorptive interactions[4,5] further adding to the molecular size retardation character of the po-

rous silica particles. Beaded cross-linked agarose will withstand less pressure than silica but will have less of these solute-matrix interactions although other solute electrostatic, adsorptive and absorptive interactions with the polysaccharide matrix may occur.

This investigation demonstrates that many of the considerations regarding electrostatic and adsorptive interactions are non-existent or are minimal when a mixture of MW HPLC marker proteins selected to elute over the range from 12 500 to 290 000 are chromatographed in buffers of near physiologic pH and ionic strength on alcohol derivatized silica *versus* cross-linked agarose as represented respectively by the TSK 3000 SWG and the Superose 12 columns. The similar separation of the five MW HPLC marker protein standards on the two matrices in the presence or absence of MW 3000 polyethylene glycol also indicates that previous concerns of solute adsorptive interactions with the matrix alcohol groups[2,3] are not universal. It also demonstrates that the presence of low-molecular-weight polyethylene glycol in the solvent does not necessarily increase the solute retention time, a concern previously raised during the preparation of leukoregulin[1].

Electrostatic, adsorptive and other solute–matrix interactions may be important in generating the diversity in the elution profiles of the oligomeric lectin preparations on the silica, zirconia stabilized silica and agarose columns observed at pH 7.4 in this investigation. Glycoproteins, for example, in addition to a propensity for hydrophobic and electrostatic matrix interaction due to their amino acid composition also have as a result of their glycosidic side chains an additional capacity for interaction with the column matrices. The interactions, moreover, may be quite different on alcohol derivatized silica compared to polysaccharide based matrices due both to the specificity and strength of the carbohydrate binding affinity of each lectin and the interactions resulting from the combined amino acid and glycosidic structure of each lectin. Solute–matrix interactions of this nature will result in a further retardation of the solute as it percolates through the column matrix. The effect is to increase the retention time and produce an apparently smaller molecular size for the component on the MW calibrated column than indicated by non-dissociating PAGE. For small macromolecular components if the solute–matrix interactions are large the retardation may be great enough to result in components with apparent molecular sizes based upon retention time that are less than those indicated by dissociating-SDS-PAGE as occurred for several of the lectins in this investigation. This is not an unexpected result of molecular sizing chromatography under non-denaturing conditions[2–4] and in some instances may actually facilitate the separation of biopolymers.

Differential electrostatic, adsorptive, and absorptive interactions can be valuable in the separation of two similarly sized macromolecules under conditions of near physiologic ionic strength and pH. As a result of these potential diverse and complex solute–matrix interactions, however, no single matrix should be considered as generally superior. None is also superior for recovery of leukoregulin bioactivity since 35% of the proliferation inhibitory activity was recovered from the zirconia stabilized silica and 90% of the activity was recovered from the silica and cross-linked agarose matrix columns. When buffers with a pH of greater than 7 are necessary, the cross-linked agarose matrix may be the best choice for repeated use since unlike the "non-stabilized" silica it will not be dissolved by the solvent. Each of the silica, zirconia stabilized silica, and cross-linked agarose molecular sizing matrices, however, can

separate diverse molecular sized proteins in the MW range from approximately 30 000 to 300 000 at pH 7.4 and will be useful to investigators seeking to isolate and characterize biologically active molecules.

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